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(54) Fermentative production of vitamin B6

(57) A process for producting vitamin B_6 comprises cultivating a microorganism belonging to the genus Rhizobium and being capable of producing vitamin B_6 in a culture medium under aerobic conditions, and separating the resulting vitamin B_6 from the fermentation broth. As well as containing assimilable carbon sources, digestible nitrogen sources, inorganic salts and other nutrients necessary for the growth of the microorganism, the culture medium preferably additionally contains pyruvate, D-glyceraldehyde, glycolaldehyde, glycine, 1-deoxy-D-threo-pentulose, 4-hydroxy-L-threonine or a mixture thereof. This process affords high yields of vitamin B_6, a vitamin essential for the nutrition of animals, plants and microorganisms and useful as a medicine and in foodstuffs.

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Description

This invention relates to a process for the production of vitamin B_6 by fermentation. "Vitamin B_6" as used in the present application includes pyridoxol, pyridoxal and pyridoxamine.

Vitamin B_6 is essential for the nutrition of animals, plants and microorganisms, and is used as a medicine and in foodstuffs. The object of the present invention is to provide a fermentative production of vitamin B_6 of high efficiently

There are many studies on the production of vitamin B_6, and various microorganisms belonging to the genera Saccharomyces, Pichia, Klebsiella, Achromobacter, Bacillus and Flavobacterium are known to produce vitamin B_6. But the accumulation of a large amount of vitamin B_6 by the microorganisms belonging to the genus Rhizobium has never been reported.

The present invention makes it is possible to produce vitamin B_6 in high yield. It has been found that the microorganisms belonging to the genus Rhizobium are capable of accumulating a large amount of vitamin B_6 in the culture broth that can be recovered therefrom in a desired purity.

The present invention is thus concerned with a process for producing vitamin B_6 which comprises cultivating a microorganism belonging to the genus Rhizobium and being capable of producing vitamin B_6 in a culture medium under aerobic conditions, and separating the resulting vitamin B_6 from the fermentation broth.

The content of vitamin B_6 in a fermentation broth can be assayed with Saccharomyces carlsbergensis ATCC 9080 [The Analysis of Nutrients in Foods, Academic Press, London, 224-227 (1978)], and the content of vitamin B_6 components such as pyridoxol, pyridoxal and pyridoxamine in a fermentation broth can also be measured separately by high performance liquid chromatography [Vitamin, 63, 349-369 (1989)].

For carrying out the present invention, microorganisms belonging to the genus Rhizobium are incubated in a culture medium containing assimilable carbon sources, digestible nitrogen sources, inorganic salts and other nutrients necessary for the growth of the microorganism. As the carbon source, for example, glucose, fructose, lactose, galactose, sucrose, maltose, starch, dextrin or glycerol may be employed. As the nitrogen source, for example, peptone, soybean powder, corn steep liquor, meat extract, ammonium sulfate, ammonium nitrate, urea or mixtures thereof may be employed. Further, as the inorganic salts, sulfates, hydrochlorides or phosphates of calcium, magnesium, zinc, manganese, cobalt and iron may be employed. And, if necessary, conventional nutrient factors or an antifoaming agent such as animal oil, vegetable oil or mineral oil can also be present supplementary. The pH of the culture medium is suitably about 5.0 to about 9.0, preferably 6.5 to 7.5. The cultivation temperature is suitably about 10 to 40°C, preferably 26 to 30°C. The cultivation time is suitably about 1 to 14 days, preferably 2 to 7 days. In the cultivation, aeration and agitation usually give favorable results. The presence of pyruvate, D-glyceraldehyde, glycolaldehyde, glycine, 1-deoxy-D-threo-pentulose, 4-hydroxy-L-threonine or an appropriate combination thereof in the medium gives more favorable results for the vitamin B_6 titer and is therefore preferred. The combination of 1-deoxy-D-threo-pentulose and 4-hydroxy-L-threonine is particularly effective as such a supplement for the production of vitamin B_6. The production of vitamin B_6 can also be achieved by incubating cells of microorganisms belonging to the genus Rhizobium separated from the culture broth in a buffer of proper pH value with appropriate combination of pyruvate, D-glyceraldehyde, glycolaldehyde, glycine, 1-deoxy-D-threo-pentulose and 4-hydroxy-L-threonine. After the cultivation, produced vitamin B_6 may be separated from the culture broth and purified. For this purpose, a process generally used for isolating product from the culture broth may be applied by utilizing various properties of vitamin B_6. Thus, for example, after the cells have been removed from the culture broth, the desired substance in the filtrate is purified using an ion exchange resin or similar means. After the elution, the desired product is recrystallized from alcohol.

The microorganism used according to the present invention includes all strains belonging to the genus Rhizobium which are capable of producing vitamin B_6 and which are preserved in a public depository (culture collection) for availability to anyone upon request, such as the Institute of Fermentation Osaka, Japan (IFO), the Ministry of Agriculture, Forestry and Fishery, Japan (MAFF) or the Institute of Applied Microbiology, the University of Tokyo, Japan (IAM); Example of such deposited strains are Rhizobium meliloti IFO 14782, Rhizobium meliloti MAFF 303040, Rhizobium meliloti MAFF 303047, Rhizobium meliloti MAFF 303097, Rhizobium tropici IFO 15247, Rhizobium huakuii IFO 15243, Rhizobium leguminosarum IFO 14778, Rhizobium galegae IFO 14965, Rhizobium fredii IFO 14780, Rhizobium loti IFO 14998, Rhizobium sp. IAM 13623 and Rhizobium sp. IAM 13631. Among these strains of the genus Rhizobium particularly preferred ones are Rhizobium meliloti IFO 14782 and Rhizobium tropici IFO 15247. The strains Rhizobium meliloti IFO 14782 and Rhizobium tropici IFO 15247 were also deposited at the DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) in Göttingen, Germany under DSM No. 10226 and No. 10227, respectively, on September 4, 1995.

The present invention will be illustrated in more detail by the following Examples; however, it should be understood that the present invention is not limited to these particular Examples.

Example 1

A loopful of cells of Rhizobium meliloti IFO 14782 (DSM No. 10226) grown on the agar medium composed of 0.1%

yeast extract (Difco), 0.5% mannitol, 0.07% K_2HPO_4, 0.01% KH_2PO_4, 0.1% $MgSO_4 \cdot 7H_2O$ and 1.5% agar (pH7.0) was inoculated into a tube containing 5 ml of the seed medium consisting of 1% glucose, 0.5% polypeptone (Nippon Seiyaku Co., Japan), 0.2% yeast extract (Difco), 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $MnSO_4 \cdot 5H_2O$ and 0.001% $FeSO_4 \cdot 7H_2O$, and then the tube was shaken on a reciprocal shaker (285 rpm) at 28°C for 17 hours. 4 ml of the seed culture were inoculated into a 500-ml flask with 2 baffles containing 200 ml of the culture medium comprising 4% glucose, 4% polypeptone S (Nippon Seiyaku Co., Japan), 0.8% yeast extract (Difco), 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% $MnSO_4 \cdot 5H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$ and one drop of antifoam CA-115 (Nippon Yushi Co., Japan), and then the flask was cultured with rotary shaking (180 rpm) at 28°C. After cultivation for 168 hours, the content of vitamin B_6 in the supernatant of the culture broth was assayed by the turbidity method with Saccharomyces carlsbergensis ATCC 9080 as described below. The supernatant and standard solutions of pyridoxol (0-100 mg per liter) were serially diluted to 1.731×10^{-4} in distilled water. 200 µl of the diluted solution, 1.5 ml of distilled water and 40 µl of 1.155 $_N$ H_2SO_4 were added to tubes in this order. After autoclaving at 120°C for 20 minutes, 1.5 ml of the assay medium for vitamin B_6 (Nissui Co., Japan) containing Saccharomyces carlsbergensis ATCC 9080 were added to the tubes, and incubated with an angle of 30° at 28°C. After incubation for 17 hours, the cell growth was stopped by adding 5 ml of 0.2 $_N$ hydrochloric acid, and then the absorbance of the samples was measured at 660 nm. The amount of vitamin B_6 in a sample was determined by comparing the turbidity of the sample with the standard growth curve of Saccharomyces carlsbergensis ATCC 9080. As a result, the supernatant of 168 hours culture broth contained 84 mg of vitamin B_6 per liter. Furthermore, after 100 µl of 4'-deoxy-pyridoxol (100 mg per liter) as internal substance had been added to 400 µl of the supernatant of 168 hours culture broth, the mixture was analyzed on a Capcell pak C_{18} SG120 column (4.6 x 250 mm, Shiseido Co., Japan) with the mixed solvent of 0.1M sodium perchlorate, 0.1M potassium phosphate and 2% acetonitrile (pH 3.5) at a flow rate of 1.0 ml/minute at λ = 292 nm by the HPLC system consisting of a Waters Model 600E system controller, a Waters Model 600F pump, a Waters Model 991J photodiode array detector, a Waters Model 700 satellite WISP sample injector and a Waters 5200 printer. As a result, it was found that the supernatant contained 78.6 mg of pyridoxol per liter.

Example 2

In a similar manner as described in Example 1, Rhizobium meliloti MAFF 303040, Rhizobium meliloti MAFF 303047, Rhizobium meliloti MAFF 303097, Rhizobium huakuii IFO 15243, Rhizobium leguminosarum IFO 14778, Rhizobium tropici IFO 15247 (DSM No. 10227), Rhizobium galegae IFO 14965, Rhizobium fredii IFO 14780, Rhizobium loti IFO 14998, Rhizobium sp. IAM 13623 and Rhizobium sp. IAM 13631 were cultivated. After cultivation in each case for 168 hours, the content of vitamin B_6 in the supernatant of the culture broth was assayed by the turbidity method with Saccharomyces carlsbergensis ATCC 9080. The results are shown in Table 1.

Table 1

Production of Vitamin B_6 by Rhizobium Strains	
Rhizobium strain	Vitamin B_6 (mg/L)
Rhizobium meliloti MAFF 303040	10.8
Rhizobium meliloti MAFF 303047	30.5
Rhizobium meliloti MAFF 303097	11.6
Rhizobium huakii IFO 15243	19.7
Rhizobium leguminosarum IFO 14778	9.85
Rhizobium tropici IFO 15247	9.00
Rhizobium galegae IFO 14965	7.81
Rhizobium fredii IFO 14780	4.02
Rhizobium loti IFO 14998	10.3
Rhizobium sp. IAM 13623	6.70
Rhizobium sp. IAM 13631	5.11

Example 3

Vitamin B_6 was recovered from the culture broth of Rhizobium meliloti IFO 14782 (DSM No. 10226) prepared under the same culturing conditions as described in Example 1. The vitamin B_6 concentration at each purification step was followed by the turbidity method with Saccharomyces carlsbergensis ATCC 9080. One liter of the 168 hours culture broth was centrifuged at 8,000 rpm for 10 minutes. The pH of the resultant supernatant was adjusted to 3.1 with 1N hydrochloric acid, and then the supernatant was applied to a column (3.6 x 40 cm) packed with 350 ml of Amberlite CG 120 (H^+ form, 100-200 mesh, Organo Co. Ltd). The column was washed with 300 ml of deionized water and then eluted with 5% ammonium hydroxide. The vitamin B_6 fractions were concentrated under reduced pressure. The residue thus obtained was dissolved in 10 ml of 0.01 M ammonium formate (pH 3.2), and the solution was charged on a column (2.4 x 40 cm) packed with 180 ml of Dowex 50w x 8 (ammonium form, 200-400 mesh, Dow Chemical Co. Ltd., U.S.A.), and then washed with 200 ml of 0.01 M ammonium formate (pH 3.2). The column was then developed with 200 ml of the starting buffer of 0.05 M ammonium formate (pH 4.25) and followed by the linear gradient of 200 ml each of 0.05 and 0.5 M ammonium formate (pH 7.0) buffer. The chromatogram gave one major and 2 minor peaks having the growth activity against Saccharomyces carlsbergensis ATCC 9080. The fraction of the major peak was concentrated to small volume under reduced pressure, the pH of the solution was adjusted to 3.1 with 1N hydrochloric acid, and then the solution was applied to a column (1.8 x 40 cm) packed with 75 ml of Amberlite CG 120 (H^+ form, 100-200 mesh). The column was washed with 150 ml of deionized water and then eluted with 5% ammonium hydroxide. The fractions having the growth activity against Saccharomyces carlsbergensis ATCC 9080 were concentrated under reduced pressure. After the solid residue had been dissolved in a small amount of hot ethanol, the solution was kept standing at 4°C overnight. The resultant precipitates were collected by filtration and dried in vacuo to obtain 51 mg of crude crystals. These were recrystallized from ethanol to obtain 44 mg of white crystals having a melting point of 160°C. The infrared absorption, UV absorption and NMR spectra of the product coincided with those of authentic pyridoxol. On the other hand, two minor peaks having the growth activity against Saccharomyces carlsbergensis ATCC 9080 were analyzed by HPLC under the analytical conditions as described in Example 1, and were identified as pyridoxamine and pyridoxal, respectively.

Example 4

Seed culture of Rhizobium tropici IFO 15247 (DSM No. 10227) was prepared under the same culturing conditions as described in Example 1. 4 ml of the seed culture were inoculated into two 500-ml flasks containing 200 ml of the culture medium comprising 2% glucose, 1% polypeptone, 0.2% yeast extract, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% $MnSO_4 \cdot 5H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$ and one drop of antifoam CA-115. Further, 4 ml of the sterilized solution containing 1% 1-deoxy-D-threo-pentulose (referred to as DTP hereafter) and 1% 4-hydroxy-L-threonine (referred to as HT hereafter) were added to one flask, and 4 ml of sterilized water to the other. Both flasks were shaken on a rotary shaker (180 rpm) at 28°C. After cultivation for 96 hours at 28°C, the content of vitamin B_6 in the supernatant of the culture broth was assayed by the turbidity method with Saccharomyces carlsbergensis ATCC 9080 as described in Example 1. As summarized in Table 2, 45 mg of vitamin B_6 per liter were produced in the flask containing the medium supplemented with DTP and HT, whereas 3.16 mg of vitamin B_6 per liter were produced in the flask containing the medium without DTP and HT. Furthermore, from one liter of the 96 hours culture broth in the medium supplemented with 0.02% each DTP and HT, the product was isolated by the same method as described in Example 3 to obtain 19.3 mg of white crystals having a melting point of 159.5°C. The infrared absorption, UV absorption and NMR spectra of the product coincided with those of authentic pyridoxol.

Table 2

Production of Vitamin B_6 by Rhizobium tropici IFO 15247 (DSM No. 10227)	
Medium	Vitamin B_6 (mg/L)
Medium supplemented with DTP and HT	45.0
Medium without DTP and HT	3.16
DTP: 1-deoxy-D-threo-pentulose, HT: 4-hydroxy-L-threonine	

Example 5

Rhizobium meliloti IFO 14782 (DSM No. 10226) was cultivated under the same culturing conditions as described in Example 1. After cultivation for 72 hours at 28°C, the cells were collected by centrifugation at 8,000 rpm for 10 minutes, washed twice with 100 ml of sterile 0.85% sodium chloride solution and suspended in 88 ml of sterile distilled water. The vitamin B_6 production was carried out with reciprocal shaking (285 rpm) at 28°C in a tube containing 10 ml of 0.1 M Tris-HCl buffer (pH 8.0) composed of 0.02% DTP, 0.24% glycolaldehyde, 0.24% glycine and the cell suspension (final optical density at 600 nm = 20/ml). After shaking for 24 hours at 28°C, vitamin B_6 in the supernatant of the reaction mixture was determined by HPLC under the analytical conditions as described in Example 1. As summarized in Table 3, 9.66 mg of pyridoxol per liter were produced from DTP, glycolaldehyde and glycine.

Table 3

Production of Pyridoxol from DTP, Glycolaldehyde and Glycine by Rhizobium meliloti IFO 14782 (DSM No. 10226)	
Substrate	Pyridoxol (mg/L)
DTP + Glycolaldehyde + Glycine	9.66
None	0
DTP: 1-deoxy-D-threo-pentulose	

Example 6

The washed cell suspension of Rhizobium meliloti IFO 14782 (DSM No. 10226) was prepared by the same method as described in Example 5. The vitamin B_6 production was carried out with reciprocal shaking (285 rpm) at 28°C in a tube containing 10 ml of 0.1 M Tris-HCl buffer (pH 7.6) comprising 0.24% pyruvate, 0.24% D-glyceraldehyde, and 0.02% HT and the washed cell suspension (final optical density at 600 nm = 20/ml). After shaking for 24 hours at 28°C, vitamin B_6 in the supernatant of the reaction mixture was determined by HPLC under the analytical conditions as described in Example 1. As summarized in Table 4, 10.1 mg of pyridoxol per liter were produced from pyruvate, D-glyceraldehyde and HT.

Table 4

Production of Pyridoxol from Pyruvate, D-Glyceraldehyde, and HT by Rhizobium meliloti IFO 14782 (DSM No. 10226)	
Substrate	Pyridoxol (mg/L)
pyruvate + D-glyceraldehyde + HT	10.1
None	0
HT: 4-hydroxy-L-threonine	

Example 7

The washed cell suspension of Rhizobium meliloti IFO 14782 (DSM No. 10226) was prepared by the same method as described in Example 5. The vitamin B_6 production was carried out with reciprocal shaking at 285 rpm at 28°C in a tube containing 10 ml of 0.1 M Tris-HCl buffer (pH 8.0) comprising 0.24% pyruvate, 0.24% D-glyceraldehyde, 0.24% glycolaldehyde, 0.24% glycine and the washed cell suspension (final optical density at 600 nm = 20/ml). After incubation for 24 hours at 28°C, vitamin B_6 in the supernatant of the reaction mixture was determined by HPLC under the analytical conditions as described in Example 1. As summarized in Table 5, 9.66 mg of pyridoxol per liter were produced from

pyruvate, D-glyceraldehyde, glycolaldehyde and glycine.

Table 5

Production of Pyridoxol from Pyruvate, D-Glyceraldehyde, Glycolaldehyde and Glycine by Rhizobium meliloti IFO 14782 (DSM No. 10226)	
Substrate	Pyridoxol (mg/L)
pyruvate + D-glyceraldehyde + glycolaldehyde + glycine	9.66
None	0

Claims

1. A process for producing vitamin B_6 which comprises cultivating a microorganism belonging to the genus Rhizobium and being capable of producing vitamin B_6 in a culture medium under aerobic conditions, and separating the resulting vitamin B_6 from the fermentation broth.

2. A process according to claim 1, wherein the cultivation is carried out in a medium containing assimilable carbon sources, digestible nitrogen sources, inorganic salts and other nutrients necessary for the growth of the microorganism, and in the presence of pyruvate, D-glyceraldehyde, glycolaldehyde, glycine, 1-deoxy-D-threo-pentulose, 4-hydroxy-threonine or a mixture thereof.

3. A process according to claim 2, wherein the cultivation is carried out in a medium containing assimilable carbon sources, digestible nitrogen sources, inorganic salts and other nutrients necessary for the growth of the microorganism, and in the presence of 1-deoxy-D-threo-pentulose and 4-hydroxy-L-threonine.

4. A process according to any one of claims 1 to 3, wherein the cultivation is carried out at a pH value of about 5.0 to about 9.0.

5. A process according to claim 4, wherein the cultivation is carried out at a pH value of 6.5 to 7.5.

6. A process according to any one of claims 1 to 5, wherein the cultivation is carried out at a temperature of about 10 to 40°C.

7. A process according to claim 6, wherein the cultivation is carried out at a temperature of 26 to 30°C.

8. A process according to any one of claims 1 to 7, wherein the cultivation is carried out for 1 to 14 days.

9. A process according to claim 8, wherein the cultivation is carried out for 2 to 7 days.

10. A process according to any one of claims 1 to 9, wherein the microorganism is Rhizobium meliloti IFO 14782 (DSM No. 10226) or Rhizobium tropici IFO 15247 (DSM No. 10227).

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DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
X	CHEMICAL ABSTRACTS, vol. 88, no. 3, 16 January 1978 Columbus, Ohio, US; abstract no. 18836, BEGISHVILI, TS. K. ET AL: "Rhizobia - producers of B2 and B6 vitamins" page 299; column left; XP002055627 * abstract * & SOOBSHCH. AKAD. NAUK GRUZ. SSR (1977), 87(2), 465-7 CODEN: SAKNAH, 1977, ---	1	C12P17/12
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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	4 March 1998	Macchia, G

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X	CHEMICAL ABSTRACTS, vol. 83, no. 1, 7 July 1975 Columbus, Ohio, US; abstract no. 4617, SAMTSEVICH, S. A. ET AL: "Effect of the carbon source in the medium on vitamin synthesis by rhizobial bacteria of lupine" page 411; column right; XP002055630 * abstract * & EKOL. POCHV. MIKROORG. (1974), 3-6. EDITOR(S): SANTSEVICH, S. A. PUBLISHER: "NAUKA I TEKHNIKA", MINSK, USSR. CODEN: 30ALAA, 1974, --- -/--	1	TECHNICAL FIELDS SEARCHED (Int.Cl.6)

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A	DATABASE WPI Section Ch, Week 7931 Derwent Publications Ltd., London, GB; Class C04, AN 79-57755B XP002055631 & SU 629 204 A (UKR FARMING RES) , 11 September 1978 * abstract * -----	1	

TECHNICAL FIELDS SEARCHED (Int.Cl.6)

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